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1 **A role of enhanced receptor engagement in the evolution of a pandemic acute haemorrhagic**
2 **conjunctivitis virus**
3

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Acute haemorrhagic conjunctivitis (AHC) is a painful, contagious eye disease, with millions of cases in the last decades. Coxsackievirus A24 (CV-A24) was not originally associated with human disease, but in 1970 a pathogenic "variant" (CV-A24v) emerged, which is now the main cause of AHC. Initially, this "variant" circulated only in Southeast Asia, but it later spread worldwide, accounting for numerous AHC outbreaks and two pandemics. While both CV-A24 "variant" and "non-variant" strains still circulate in humans, only "variant" strains cause AHC for reasons that are yet unknown. Since receptors are important determinants of viral tropism, we set out to map the CV-A24 receptor repertoire and establish whether changes in receptor preference have led to the increased pathogenicity and rapid spread of CV-A24v. Here, we identify ICAM-1 as an essential receptor for both AHC-causing and non-AHC strains. We provide the first high-resolution cryo-EM structure of a virus-ICAM-1 complex, which revealed critical ICAM-1-binding residues. These data could help identify a possible conserved mode of receptor engagement among ICAM-1-binding enteroviruses and rhinoviruses. Moreover, we identify a single capsid substitution that has been adopted by all pandemic CV-A24v strains and we reveal that this adaptation enhances the capacity of CV-A24v to bind sialic acid. Our data elucidate the CV-A24v receptor repertoire and point to a role of enhanced receptor engagement in the adaptation to the eye, possibly enabling pandemic spread.

Significance statement

Acute haemorrhagic conjunctivitis (AHC) is a painful and highly contagious infection of the eye, with reported incidence rates of up to 48%. No drugs or vaccines are available for treatment or prevention of AHC. Coxsackievirus A24 variant (CV-A24v) is the main etiological agent of AHC, being responsible for >10 million AHC cases worldwide during the last decades. We have identified the CV-A24v protein receptor and determined the high-resolution structure of the virus-receptor complex. Furthermore, we found that an adaptation which enhances binding to the receptor sialic acid may have contributed to the pathogenicity and pandemic nature of CV-A24v. These findings highlight the importance of sialic acid for viruses with ocular tropism, such as influenza A virus and several adenoviruses.

Introduction

Acute haemorrhagic conjunctivitis (AHC) is characterized by a sudden onset of ocular pain, swelling, watering and extensive subconjunctival haemorrhaging. Since AHC is highly contagious, it spreads rapidly and can affect up to 48% of a population in a single outbreak (1, 2). Coxsackievirus A24 variant (CV-A24v), belonging to the genus Enterovirus within the family Picornaviridae, is the main etiological agent of AHC, being responsible for >10 million AHC cases worldwide during the last decades (2, 3). Coxsackievirus A24 (CV-A24) was first isolated in South Africa in 1951 (4), but was not associated with disease in humans. However, in 1970, a pathogenic variant of CV-A24 (CV-A24v) emerged that was responsible for a large AHC outbreak in Singapore (5, 6). Initially, this AHC-causing variant circulated only in Southeast Asia, but later spread worldwide, suddenly causing numerous explosive AHC outbreaks and two pandemics, which started in 1985 and 2002 (7–9). Although both the CV-A24 variant and non-variant strains are being detected in clinical samples worldwide, only strains belonging to the "variant" clade are associated with AHC. As yet, the molecular mechanism underlying this difference in pathogenicity is unknown. Also, it remains unclear what has led to the sudden onset of CV-A24v pandemics.

Receptor choice or the interplay between different receptors can be important determinants of viral tropism and pathogenicity. Most enteroviruses rely on an uncoating receptor that triggers destabilizing rearrangements of the capsid proteins, resulting in the formation of a capsid-mediated pore in the endosomal membrane through which the viral genome is delivered into the cytoplasm (10). In addition to uncoating receptors, many enteroviruses employ one or more attachment receptors that do not participate in uncoating but facilitate cell binding. Previous reports have shown that sialic acid (Sia) plays a role in infection of CV-A24v (11). Despite these findings, it remains unclear whether CV-A24v requires additional cell surface receptors and whether differences in receptor preference exist between AHC- and non-AHC-causing CV-A24 strains.

In this study, we set out to identify a potential protein receptor and to investigate if possible changes in receptor requirements can explain the pathogenic and pandemic nature of AHC-causing CV-A24v strains. We identify ICAM-1 as an essential receptor for both AHC- and non-AHC-causing CV-A24 strains and provide the first high-resolution cryo-EM structure of a virus in complex with ICAM-1, revealing in detail the interactions between CV-A24v and its receptor. Moreover, by combining structural data, phylogenetic analysis and reverse genetics, we identify a single amino acid substitution in the viral capsid that provides an improved Sia binding capacity and has been adopted exclusively by pandemic CV-A24 "variant" strains. These findings point towards a role of enhanced Sia binding in the adaptation of CV-A24v to the eye, which may have been a prerequisite for its pandemic potential.

98   **Results**

99

100   **ICAM-1 is an essential CV-A24v receptor**

101   CV-A24v can bind Sia, but Sia was not found to initiate uncoating (12), suggesting that CV-A24v

102   employs an additional, yet unknown, receptor. In order to identify this receptor, we performed

103   neutralization assays with a CV-A24v clinical isolate in Sia-deficient HAP1 CMAS^{KO} cells (13), using

104   soluble receptor domains (VLDL-R) or antibodies targeting various known enterovirus receptors

105   (ICAM-1, DAF, integrin α2, PVR, CAR, integrin αvβ3, and PSGL-1) (**Fig. 1A**). The ability of these

106   ligands to suppress receptor binding was confirmed using positive control viruses (**Fig. S1A**). Only the

107   antibody against ICAM-1, a cell adhesion molecule consisting of five Ig-like domains (14) (**Fig. 1B**),

108   blocked CV-A24v infection (**Fig. 1A**). To determine whether ICAM-1 is essential for CV-A24v, we

109   knocked out ICAM-1 in various cell types by CRISPR/Cas9 genome editing (**Fig. 1B**). The ICAM-1

110   knockout (ICAM-1^{KO}) genotype was verified by sequence analysis (**Fig. S1B**) and by assessing the

111   susceptibility of ICAM-1^{KO} cells to infection with ICAM-1-dependent coxsackievirus A21 (CV-A21)

112   (15) and rhinovirus B14 (RV-B14) (16), and CAR-dependent coxsackievirus B3 (CV-B3) (17) (**Fig. 1**

113   **F and G and S1 C-E**). CV-A24v infection was reduced by ICAM-1 knockout in HAP1 CMAS^{KO} cells

114   (**Fig. S1C**), wt HAP1 cells (**Fig. S1D**), as well as HeLa cells (**Fig. 1C**), and was rescued by transfecting

115   ICAM1 cDNA (**Fig. 1C**). Bio-layer interferometry (**Fig. 1D**) and CV-A24v neutralization with soluble

116   ICAM-1 ectodomain in primary conjunctival cells (**Fig. 1E**) pointed to a direct interaction between

117   CV-A24v and ICAM-1. Analysis of virus production in HeLa cells (**Fig. 1F**) and analysis of cytopathic

118   effect induction in HeLa (**Fig. 1G**) or human corneal epithelial (HCE) cells (**Fig. S1E**) showed that

119   several CV-A24v clinical isolates, the non-variant prototype strain Joseph, and a non-variant clinical

120   isolate all require ICAM-1 for infection. Sialylation of ICAM-1 was not found to be essential for

121   interaction with CV-A24v and CV-A24 Joseph, as shown by bio-layer interferometry (**Fig. S1F**).

122   Taken together, these data indicate that ICAM-1 functions as an essential receptor for AHC-causing

123   variant strains as well as non-variant strains.

124

125   **The structure of CV-A24v in complex with ICAM-1**

126   To elucidate the molecular interaction of CV-A24v with ICAM-1, we determined the structure of a

127   complex between purified virions and a recombinant D1-D2 fragment of ICAM-1 (**Fig. 2 A and B and**

128   **Table S1**). CV-A24v virions were immobilized on a lacey carbon support after which D1-D2 was

129   added 30 seconds at 8 °C prior to blotting and plunge freezing. This rapid on-grid binding approach

130   allowed us to capture the cryo-EM structure of CV-A24v with bound D1-D2, whereas longer

131   incubation resulted in loss of particle integrity, probably due to receptor-mediated destabilization of the

132   particle. The global resolution of the EM density is 3.9 Å (**Fig. S2 A-D**), the resolution of the viral

133   capsid proteins ~3.6-3.8 Å and that of the capsid-interfacing region of ICAM-1 D1 ~3.7 Å (**Fig. S2 E-**

134   **H**), sufficient to resolve bulky side chains in the electron density map (**Fig. 2C**). The surface of an

135   enterovirus particle is comprised of 60 copies of the structural proteins VP1, VP2, and VP3 (**Fig. 2A**).

136   A depression encircling each five-fold axis of symmetry forms the so-called 'canyon', which is the

137   binding site for all uncoating receptors identified to date. The structure shows that the D1 domain binds

138   in the canyon at the quasi-threefold axis of the capsid (**Fig. 2B**). In contrast to earlier studies, the

139 interface between the capsid and ICAM-1 D1 is well defined in the final EM density map, enabling us
140 to resolve secondary structure and the majority of side chains in this portion of the structure. This, in
141 turn, allowed us to calculate an accurate footprint of the D1 component of the receptor on the virus
142 surface, shown as a 'roadmap' projection (**Fig. 2D**). The specificity in the interaction between ICAM-1
143 and CV-A24v is largely driven by electrostatic complementarity (**Fig. 2E and S2I**). Specifically, Lys[29]
144 (FG loop) of ICAM-1 interacts with VP3 Asp[181] and VP1 Asp[238] (**Fig. 2F and S2J**). In addition, Asp[71]
145 on the tip of the FG loop of ICAM-1 forms a salt bridge with VP1 Arg[168]. Finally, Lys[50] (strand C) and
146 Lys[39] (strand D) of ICAM-1 form salt bridges with VP1 Asp[228] and Asp[225] (GH loop). Additional
147 interactions include a hydrogen bonding network that tethers the DE loop of ICAM-1 D1 (Arg[49], Asn[47]
148 and Pro[45]) to VP1 (Tyr[230]) and VP2 (Ala[138], Lys[139] and Thr[140]) (**Fig. S2K**). Many of these residues are
149 conserved among other ICAM-1-binding viruses belonging to the enterovirus C species, suggesting a
150 conserved mode of interaction with ICAM-1 (18). On the contrary, only VP1 Asp[238] and VP3 Asp[181]
151 are present in ICAM-1-binding rhinoviruses.

152

153 **Sia is an attachment receptor supporting ICAM-1-mediated CV-A24v infection**
154 In addition to their uncoating receptor, several enteroviruses exploit additional receptors to enhance cell
155 attachment. Since previous studies identified a role of Sia for CV-A24v (11, 12), we set out to
156 investigate how Sia and ICAM-1 cooperate during CV-A24v infection. Removal of cell-surface Sia by
157 CMAS knockout or NA treatment revealed that Sia supports CV-A24v infection (**Fig. 3A and S3A**).
158 The role of Sia was more prominent when infection was performed on ice, compared to infection at
159 37°C (**Fig. 3A**). This may be due to stabilization of the interaction at low temperature, as was
160 previously described for several other Sia-binding viruses (19, 20). NA treatment of several cell types
161 revealed that Sia supports CV-A24v infection of HCE and HAP1 cells, but not of HeLa cells (**Fig. 3B**).
162 This difference might result from variability in the expression levels of ICAM-1/Sia or in the types of
163 Sia expressed on cells. Sia removal from HCE cells (**Fig. 3C and 3D and S3B**) or HAP1 cells (**Fig.
164 S3C**) incompletely inhibited CV-A24v infection, whereas knockout of ICAM-1 completely blocked
165 CV-A24v infection (**Fig. 3D**). Analysis of virus binding to HCE cells (**Fig. 3E**) and primary
166 conjunctival cells (**Fig. 3F**) showed that Sia plays a role in cell attachment of CV-A24v. Together,
167 these results show that, while ICAM-1 is the main CV-A24v receptor, Sia functions as an auxiliary
168 attachment receptor.

169

170 **Differential Sia dependency between AHC-causing strains and non-AHC strains**
171 Next, we tested whether AHC strains and non-AHC strains differ in their dependency on cell surface
172 Sia, either by treating HCE cells with NA to remove Sia (**Fig. 4A and S4A**) or with NaIO$_3$ to oxidize
173 carbohydrates (**Fig. S4B**). The AHC strains were more reliant on Sia than a non-AHC clinical isolate
174 and the non-AHC prototype strain Joseph. Comparison of VP1 residues that constitute the Sia-binding
175 site (12) showed that only one residue present in all AHC strains (VP1 Tyr[250]) was absent in the non-
176 AHC strains (VP1 Phe[250]) (**Fig. 4B**). Although a Sia-binding site lacking this residue might be
177 functional, the presence of Tyr[250] could increase the affinity by hydrogen bonding with the 5-N-acyl

178 carbonyl group in Sia (**Fig. 4C**). A similar mode of Sia stabilization is seen in other viral lectins, such
179 as coronavirus hemagglutinin-esterases (20) and influenza neuraminidases (19) (**Fig. S4C**).
180
181 **Pandemic CV-A24v strains have acquired a capsid residue that enhances sialic acid binding**
182 After its emergence in 1970 in Singapore, the pathogenic CV-A24 "variant" initially circulated only in
183 Southeast Asia but started to spread globally in 1985, causing numerous AHC outbreaks and two
184 pandemics. Concurrently, CV-A24 strains that do not belong to the "variant" clade and do not cause
185 AHC continued to circulate worldwide and are sporadically found in stool samples. To understand
186 whether the presence of Tyr^{250} correlates with the ability of CV-A24v to cause AHC, we compared the
187 Sia-binding sites between "variant" and "non-variant" strains. To classify these strains, we constructed
188 a phylogenetic tree of all available complete CV-A24 VP1 sequences (**Fig. S4D**), originating from
189 isolates collected worldwide during seven decades, including the first AHC outbreak in 1970 and the
190 two subsequent pandemics that started in 1985 and 2002 (**Fig. 4D**). This tree showed that of the 226
191 strains, 189 belong to the "variant" clade, which is delineated by the earliest AHC-causing strain
192 EH24/70 that was isolated in Singapore in 1970 (**Fig. 4E**). A comparison of amino acid frequencies in
193 the Sia-binding site revealed that most "non-variant" strains, as well as the first AHC strain EH24/70
194 have a phenylalanine at VP1 position 250, whereas all "variant" strains isolated since the first AHC
195 pandemic possess VP1 Tyr^{250} (**Fig. 4E**). Such a genetic change could result from a population bottle-
196 neck that "variant" strains may have undergone when invading a new target tissue. However, the
197 absence of this residue in the "ancestral" strain EH24/70 suggests that Tyr^{250} was not present during the
198 initial tropism switch, but was adopted by its pandemic descendants in consequence of positive
199 selection in the eye. The selective pressure to maintain this residue might be due to a more prominent
200 role of Sia for infection in the ocular tissue to which AHC strains have adapted. To establish whether
201 Tyr^{250} promotes Sia usage, we constructed an infectious cDNA clone of CV-A24v strain 110390 and
202 mutated Tyr^{250} to Phe. This mutant and the wildtype virus were equally infectious in HeLa cells, in
203 which Sia does not play a role. In HCE cells, however, wildtype CV-A24v was more infectious than
204 the $Tyr^{250}Phe$ mutant (**Fig. 4F**) and this difference was abolished by NA treatment. Consistently, the
205 wildtype virus bound better to Sia-expressing HCE cells than the $Tyr^{250}Phe$ mutant (**Fig. 4G**),
206 indicating that Tyr^{250} increases the capacity of CV-A24v to bind Sia. Together, these data demonstrate
207 that the tropism switch of CV-A24 towards the eye was followed by the acquisition of a residue that
208 enhances Sia-binding, an adaptation which coincides with the emergence of pandemic CV-A24v.
209
210 **Discussion**
211 This study provides new insights into the receptor requirements of CV-A24v and reveals that a subtle
212 change in the engagement of a secondary attachment receptor, Sia, may have promoted the adaptation
213 of a pathogen to a new niche. Although VP1 Tyr^{250} is not a prerequisite for Sia binding (**Fig. 4F**), our
214 results show that the presence of this residue can enhance Sia binding. Possibly together with other
215 mutations outside the Sia-binding site, the adoption of an enhanced Sia-binding capacity by pandemic
216 AHC-causing strains may have been a crucial evolutionary step in becoming a well-adapted ocular
217 virus that can rapidly spread worldwide and cause AHC pandemics. Sia is also a receptor for several

other ocular viruses such as adenovirus type 37, enterovirus D70 and influenza A virus (21–23), suggesting that Sia is important particularly for infection of the eye. Improved Sia-binding properties may promote CV-A24v attachment to and infection of conjunctival cells, thereby increasing the viral load in the eye and enhancing transmission. Since CV-A24v is believed to be transmitted via hand-to-eye contact, the fate of CV-A24v after the initial disposition into the eye might be largely determined by its capacity to attach to the conjunctival tissue before it is cleared by the continuous draining of tear film through the nasolacrimal duct. A weak and dynamic interaction with highly sialylated mucus proteins might facilitate this initial attachment and rapidly secure virions in the mucus layer, without eliminating virus mobility.

While the emergence of pandemic CV-A24v may have been promoted by the acquisition of VP1 Tyr[250], it remains unknown what has caused the initial adaptation of CV-A24 to the eye. The genetic change underlying this tropism switch may be a combination of multiple synergistic mutations, which could reside in the capsid-coding region, non-structural genes, or non-coding regulatory elements in the viral genome. For CV-A24v, it has been reported that the emergence of the AHC-causing CV-A24v "variant" was accompanied by a loss of amino acid diversity (**Table S2**), probably as a result of a population bottleneck (24). Consequently, numerous genetic differences exist between "variant" and "non-variant" strains. Such changes in amino acid diversity also occurred at several CV-A24 capsid sites that interact with ICAM-1 (**Table S3**), some of which may have caused subtle changes in the engagement of ICAM-1 that facilitated ocular tropism. Thus, the identification of ICAM-1-binding residues in CV-A24v may help future studies investigating whether changes in ICAM-1 binding have contributed to the earliest manifestation of AHC.

242 **Materials and Methods**

243 Extended methods and information about cell lines, viruses (including construction of CV-A24v

244 110390 infectious cDNA clones), chemicals and reagents, are described in **SI Materials and methods**.

245

246 **Electron microscopy.** Cryo-EM grids were prepared by applying 3 µl of purified CV-A24v (10

247 mg/ml) to 400 mesh lacey grids coated in a 3 nm carbon film (Agar Scientific, UK). The sample was

248 left to adsorb for 30 seconds before most of the sample was blotted away manually. On-grid binding of

249 the receptor was performed by applying 3 µl of ICAM-1 D1-D2 (9.85 mg/ml) to the pre-blotted, virus-

250 containing grid, and leaving for 30 seconds before blotting and freezing using a Leica EM GP plunge

251 freeze (Leica Microsystems) device. The Leica EM chamber temperature was set to 8 °C with 80 %

252 relative humidity and liquid ethane was used for sample vitrification. Grids were glow discharged for

253 30 seconds prior to application of the samples. CV-A24v – ICAM-1 D1-D2 data was collected on an

254 FEI Titan Krios (ABSL, University of Leeds) transmission electron microscope at 300 kV, using a total

255 electron dose of 60 e-/$Å^2$ and a magnification of 75,000x. The final calibrated object sampling was

256 1.065 Å/pixel. A total of 2643 exposures were recorded using the EPU automated acquisition software

257 on a FEI Falcon III direct electron detector operating in linear mode. Each exposure movie had a total

258 exposure of one second and contained 40 frames. Image processing, model building and refinement are

259 described in **SI Materials and methods**.

260

261 **Infectivity assays.** Cells were incubated with virus for 1 hour at 37°C or, where indicated, on ice. After

262 virus binding, cells were washed once with PBS (ice-cold PBS was used after virus binding on ice).

263 Cells were supplied with fresh medium and incubated at 37°C for a total of 8 hours, which represents a

264 single replication cycle. In virus production assays, cells were subjected to three freeze/thaw cycles and

265 virus titers were determined by end-point dilution. Crystal violet staining was performed 4 days post-

266 infection. In neutralization assays, cells were pretreated with antisera or soluble receptors for 45

267 minutes at 37°C. Unless otherwise indicated, neuraminidase treatments were performed by incubating

268 cells with a 1:30 dilution of A. urefaciens NA in serum-free medium for 1 hour at 37°C. Treatments

269 with periodate, which oxidizes vicinal diols in glycans, was performed by incubation of cells with 5

270 mM $NaIO_3$ in PBS for 1 hour on ice.

271

272 **Immunofluorescence assays.** Cells were fixed by submersion in a 4% paraformaldehyde solution for

273 15 minutes. Fixed cells were stained with 1:2000 diluted mouse monoclonal anti-dsRNA (J2; English

274 and Scientific Consulting). Cells were examined by confocal microscopy (Leica SPE-II) and the

275 number of infected cells was quantified with ImageJ.

276

277 **Virus binding assays.** HCE cells were successively incubated with NA for 1 hour at 37°C, 1% BSA in

278 PBS for 10 min on ice, virus for one hour on ice and washed three times with ice-cold PBS. RNA was

279 isolated from cells using the NucleoSpin RNA Isolation Kit (Macherey-Nagel, Ref.No. 740955.250).

280 cDNA was generated using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems,

281 Ref.No. N8080234). Quantitative PCR was performed using the Roche Lightcycler 480 SYBR Green I

282 Master kit (Roche, Ref.No. 04 887 352 001). For binding assays with HC0597 cells, CV-A24v was ^{35}S-
283 labelled as described previously (11), followed by treatment with soluble proteins for 1 h at 37°C.
284 Adherent HC0597 cells were detached (with phosphate-buffered saline [PBS]–0.05% EDTA, Merck)
285 and recovered in growth medium at 37°C. After 1h, 100.000 cells/sample were washed and diluted in
286 50µl binding buffer (HC0597 media + 0,5% Bovine serum albumin (Roche, Stockholm, Sweden) +
287 HEPES pH 7.4 (EuroClone, Milan, Italy)) before the addition of 5000 ^{35}S-labeled CVA24v virions/cell.
288 Cells were washed and the radioactivity of the cells was measured using a Wallac 1409 scintillation
289 counter (Perkin-Elmer, Waltham, MA).

290

291 **Bio-layer interferometry.** Biolayer interferometry was performed with the Octet® Red 384 (ForteBio)
292 using biotinylated ICAM-1 and LAMP-1. Streptavidin sensors were loaded with a 2 µg/ul solution of
293 each protein for 15 min. Association of viruses to the sensor was performed for 60 min.

294

295 **Phylogenetic analysis.** All available CV-A24(v) sequences spanning the entire VP1 protein were
296 downloaded from Genbank (January 1, 2017) and aligned using the ClustalW (1.6) algorithm
297 implemented in MEGA version 7, followed by manual refinement. A phylogenetic tree of VP1
298 sequences was constructed using the neighbor-joining method and the maximum composite likelihood
299 method implemented in MEGA version 7. The phylogenetic tree was visualized using Figtree version
300 1.4.3.

301

302 **Data availability.** The cryo-EM reconstruction and atomic model are deposited in the Electron
303 Microscopy Data Bank (EMDB) and Protein Data Bank (PDB) under the accession codes EMD-3880
304 and PDB-6EIT. VP1 sequences are available in Genbank under accession numbers MG272373,
305 MG272374 and MG272375.

306

314

References

1. Ghazali O, et al. (2003) An outbreak of acute haemorrhagic conjunctivitis in Melaka, Malaysia. Singapore Med J 44(10):511–516.

2. Kurokawa M, Rai SK, Ono K, Gurung R, Ruit S (2006) Viral investigation of acute hemorrhagic conjunctivitis outbreak (2003) in Nepal using molecular methods. Southeast Asian J Trop Med Public Health 37(5):904–910.

3. Oh M, et al. (2003) Acute Hemorrhagic Conjunctivitis Caused by Coxsackievirus A24 Variant, South Korea, 2002. Emerg Infect Dis 9(8):8–10.

4. Measroch V, Gear J, Faerber GI (1951) Studies in Poliomyelitis: the isolation of a coxsackie-like virus from the faeces of apparently healthy Bantu infants. South African Med J 25(25).

5. Kono R, et al. (1972) Pandemic of new type of conjunctivitis. Lancet 1(7762):1191–1194.

6. Mirkovic RR, Schmidt NJ, Yin-Murphy M, Melnick JL (1974) Enterovirus Etiology of the 1970 Singapore Epidemic of Acute Conjunctivitis. Intervirology 4:119–127.

7. Miyamura K, et al. (1988) The first epidemic of acute hemorrhagic conjunctivitis due to a coxsackievirus A24 variant in Okinawa, Japan, in 1985-1986. Japanese J Med Sci Biol 41:159–174.

8. Ishiko H, et al. (1992) Phylogenetic analysis of a coxsackievirus A24 variant: The most recent worldwide pandemic was caused by progenies of a virus prevalent around 1981. Virology 187(2):748–759.

9. Lévêque N, Huguet P, Norder H, Chomel J-J (2010) Les Enterovirus responsables de conjonctivite aiguë hémorragique. Médecine Mal Infect 40(4):212–218.

10. Rossmann MG, He Y, Kuhn RJ (2002) Picornavirus-receptor interactions. Trends Microbiol 10(7):324–331.

11. Nilsson EC, Jamshidi F, Johansson SMC, Oberste MS, Arnberg N (2008) Sialic acid is a cellular receptor for coxsackievirus A24 variant, an emerging virus with pandemic potential. J Virol 82(6):3061–8.

12. Zocher G, et al. (2014) A Sialic Acid Binding Site in a Human Picornavirus. PLoS Pathog 10(10):e1004401.

13. Baggen J, et al. (2016) Enterovirus D68 receptor requirements unveiled by haploid genetics. Proc Natl Acad Sci 113(5):1399–1404.

14. van de Stolpe A, van der Saag PT (1996) Intercellular adhesion molecule-1. J Mol Med (Berl) 74(1):13–33.

15. Shafren DR, Dorahy DJ, Greive SJ, Burns GF, Barry RD (1997) Mouse Cells Expressing Human Intercellular Adhesion Molecule-1 Are Susceptible to Infection by Coxsackievirus A21. 71(1):785–789.

16. Greve JM, et al. (1989) The major human rhinovirus receptor is ICAM-1. Cell 56(5):839–47.

17. Bergelson JM, et al. (1997) Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 275(5304):1320–1323.

18. Xiao C, et al. (2005) The crystal structure of coxsackievirus A21 and its interaction with ICAM-1. Structure 13(7):1019–33.

355   19.   Varghese JN, et al. (1997) Structural evidence for a second sialic acid binding site in avian
356   influenza virus neuraminidases. Proc Natl Acad Sci U S A 94(22):11808–12.

357   20.   Bakkers MJG, et al. (2016) Coronavirus receptor switch explained from the stereochemistry of
358   protein–carbohydrate interactions and a single mutation. Proc Natl Acad Sci:E3111–E3119.

359   21.   Arnberg N, Edlund K, Kidd AH, Wadell G (2000) Adenovirus type 37 uses sialic acid as a
360   cellular receptor. J Virol 74(1):42–8.

361   22.   Alexander D a., Dimock K (2002) Sialic Acid Functions in Enterovirus 70 Binding and
362   Infection. J Virol 76(22):11265–11272.

363   23.   Belser J a, Rota P a, Tumpey TM (2013) Ocular tropism of respiratory viruses. Microbiol Mol
364   Biol Rev 77(1):144–56.

365   24.   Smura T, et al. (2014) The Evolution of Vp1 Gene in Enterovirus C Species Sub-Group That
366   Contains Types CVA-21, CVA-24, EV-C95, EV-C96 and EV-C99. PLoS One 9(4):1–16.

367

Figure legends

370   **Figure 1.** ICAM-1 is an essential CV-A24 receptor. (**A**) Yields of infectious virus after a single
371   replication cycle. Sia-deficient HAP1 CMAS^{KO} cells were treated with antibodies against enterovirus
372   receptors (ICAM-1, DAF, integrin α2, PVR, CAR, integrin αvβ3, and PSGL-1) or soluble receptor
373   (VLDL-R) and infected at 37 °C with CV-A24v 110387. (**B**) Schematic representation of ICAM-1
374   showing immunoglobulin-like domains 1-5 (D1-D5) and the two sites targeted by CRISPR/Cas9
375   (gRNA 1 and 2). Knockout was accomplished by disrupting a 1489 bp region of ICAM1 encoding the
376   transmembrane domain (TM), either by excision of this region or by introducing a frameshift mutation.
377   (**C**) HeLa-R19 ICAM-1^{KO} transfected with plasmid encoding ICAM-1 cDNA were exposed to virus and
378   yields of infectious virus were measured after a single replication cycle. (**D**) Bio-layer interferometry
379   analysis of virus binding to either ICAM-1 or negative control receptor LAMP-1. (**E**) Relative levels of
380   CV-A24v 110390 bound to primary conjunctival cells with expanded lifespan (HC0597), in the
381   presence of ICAM-1 D1D5 or negative control receptor CAR D1. (**F**) HeLa-R19 cells were infected
382   and yields of infectious virus were determined after a single replication cycle. Dashed lines (A, C and
383   F) represent virus input levels (T=0). Error bars (A, C, E and F) represent the mean ± SEM of 3-4
384   biological replicates. (**G**) HeLa-R19 cells were infected with seven CV-A24v clinical isolates, two CV-
385   A24 "non-variant" strains and ICAM-1-dependent (CV-A21, RV-14) or CAR-dependent (CV-B3)
386   control viruses, followed by crystal violet staining of surviving cells.

387

388   **Figure 2.** The structure of CV-A24v in complex with ICAM-1. (**A**) Enlarged tilted view of density
389   denoted by a black triangle in (B), containing the corresponding atomic model. ICAM-1 D1 binds in
390   the 'canyon' located at the quasi three-fold axis. (**B**) The cryo-EM reconstruction of CV-A24v in
391   complex with ICAM-1 (D1D2) viewed down the icosahedral two-fold axis. VP1-3 (3.6σ), D1 (1σ) and
392   D2 (0.6σ). (**C**) Typical example of the cryo-EM electron density of VP1-3 and D1 domain. (**D**)
393   Radially colored isosurface representation of CV-A24v in complex with D1 (grey) viewed down the
394   icosahedral two-fold axis (left). The stereographic projection of the viral surface (right), where the
395   polar angles θ and φ represent latitude and longitude, respectively. Amino acids which interact with
396   ICAM-1 D1 are located at the floor and wall of the canyon (blue) and are circled in white. The radial
397   colouring key is shown in angstroms. (**E**) Surface representation of the EM-derived atomic model for
398   the quasi three-fold axis of CV-A24v (grey) and ICAM-1 D1 (pink), with residues forming salt bridges
399   labelled and coloured in respect to their interacting partners. The left panel shows the surface of CV-
400   A24v and the right panel shows the surface of ICAM-1 D1 that interacts with CV-A24v. Residues
401   forming salt bridges are coloured according to charge (red is negative, blue is positive) and capsid
402   residues that hydrogen bond with ICAM-1 are dark grey. (**F**) Overview of electrostatic interactions
403   shown in (E) between ICAM-1 (pink) and CV-A24v (blue) with potential hydrogen bonds denoted as
404   dotted black lines and EM density as a green mesh.

405

406   **Figure 3.** Sia is an attachment receptor supporting ICAM-1-mediated CV-A24v infection. (**A**) HCE
407   cells were treated with a mixture of A. urefaciens and V. cholerae NA (1:30), infected with CV-A24v

408 110387 on ice (0°C) or at 37°C, followed by qPCR analysis of viral RNA levels after a single

409 replication cycle. (**B**) NA-treated cells were infected with CV-A24v 110387 and yields of infectious

410 virus were determined after a single replication cycle. (**C**) NA-treated HCE cells were infected on ice

411 with CV-A24v, Sia-dependent enterovirus D68 (EV-D68) and Sia-independent CV-B3, followed by

412 staining of dsRNA (green) and nuclei (blue). Shown are representative confocal micrographs.

413 Percentage values denote mean ± SEM of three technical replicates, normalized to mock. (**D** and **E**)

414 HCE cells were treated with a mixture of A. urefaciens and V. cholerae NA (1:30) and infected on ice.

415 After incubation with virus, viral RNA levels were determined by qPCR either directly (E) or after a

416 single replication cycle (D). (**F**) Levels of radioactively labeled CV-A24v 110390 bound to NA-treated

417 (V. cholerae 10 mU/ml) primary conjunctival cells with expanded lifespan (HC0597) on ice (0°C) or at

418 37°C. Dashed lines (A, B and D) represent virus input levels (T=0). Error bars represent the mean ±

419 SEM of 3 (A, B, D and E) or 8 (F) biological replicates. P-values (A and F) were calculated by an

420 unpaired two-sided T-test; *P<0.05; **P < 0.01; ***P < 0.001.

421

422 **Figure 4.** Pandemic CV-A24v strains have acquired a capsid residue that enhances sialic acid binding.

423 (**A**) NA-treated HCE cells were infected on ice, followed by staining of infected cells with a dsRNA

424 antibody (green) and nuclei with DAPI (blue). Percentage values denote mean ± SEM of three

425 technical replicates, normalized to mock. (**B**) Amino acid sequence alignment of tested CV-A24

426 strains, with the residue unique for non-variant strains highlighted in red. (**C**) Binding site for Sia

427 (orange) in CV-A24v 110390 (PDB 4Q4X), showing that the 5-N-acyl group of Sia is stabilized by two

428 hydrogen bonds (dashed lines). Two adjacent VP1 proteins are colored grey and blue, respectively. Red

429 spheres represent water molecules. Oxygen and nitrogen atoms are colored red and blue, respectively.

430 (**D**) Geographic origins of non-AHC- (red) and AHC-causing (purple, light blue, blue) CV-A24 strains

431 of which complete VP1 sequences are available, with circle sizes proportional to the number of

432 isolates. (**E**) Phylogenetic tree of CV-A24 isolates, showing on the right side the frequencies of amino

433 acids in the Sia-binding site among the indicated number of strains. Frequency plots were generated

434 with WebLogo. Residues at VP1 position 250 are highlighted in gray. (**F**) HCE cells were treated with

435 NA and infected on ice with CV-A24v 110390 or the VP1 Y250F mutant, followed by staining of

436 dsRNA (green) and nuclei (blue). Quantifications shown at the bottom denote mean ± SEM of four

437 technical and two biological replicates. Images shown in A and F are representative confocal

438 micrographs. (**G**) HCE cells were treated with a mixture of A. urefaciens and V. cholerae NA (1:30)

439 and incubated with virus on ice, followed by qPCR analysis of bound virus. Error bars represent the

440 mean ± SEM of 3 biological replicates. P-values (F and G) were calculated by an unpaired two-sided

441 T-test; ns P ≥ 0.05; **P < 0.01; ****P < 0.0001.

A *HCE cells*
CV-A24v110387

B CV-A24v 110387

C *HCE cells*
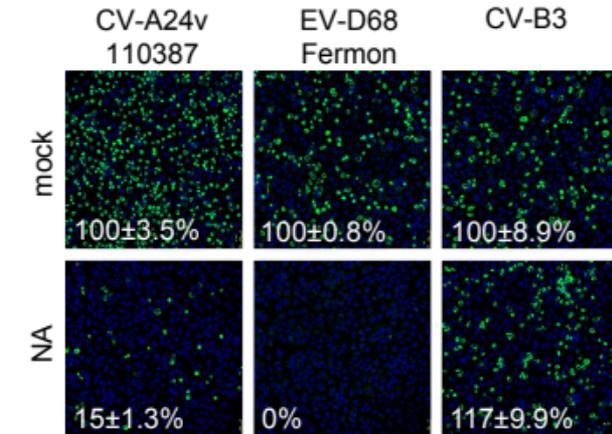
D *Virus replication in HCE*
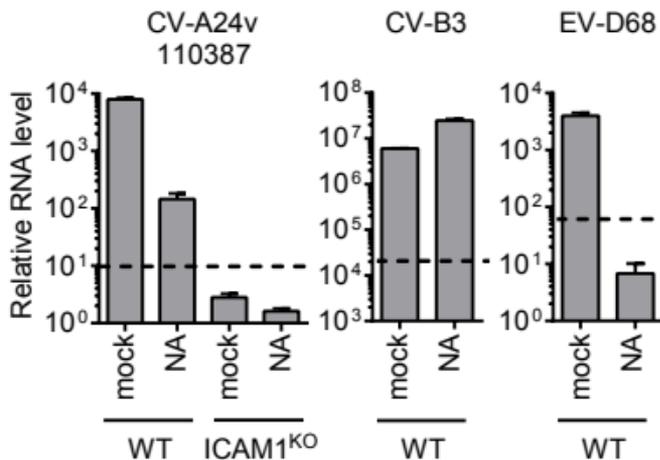
E *Virus binding to HCE*
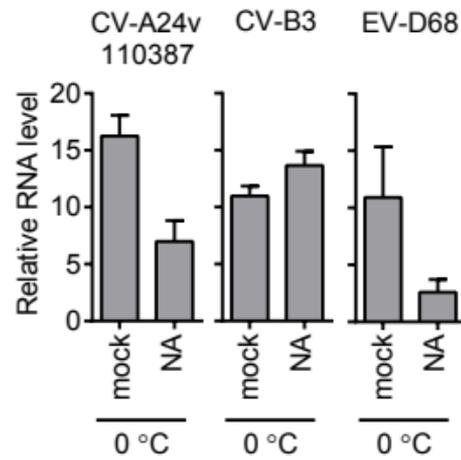
F *Virus binding to HC0597cells*
CV-A24v 110390

A

CV-A24v 110387 / CV-A24 Joseph / CV-B3

mock: 100±2.8% / 100±10.0% / 100±8.2%

NA: 15.4±2.0% / 61.8±8.2% / 153.5±11.7%

B

Sia-binding site

variant:
110387 ENFNA..TERYYTSNT..NEYNPARIT
110389 ENFNA..TERYYASNT..NEYNPARIT
110390 ENFNA..TERYYASNT..NEYNPARIT
Joseph ENFNA..TERYYASNT..NE**F**NPARIT
73-17674 ENFNA..TERYYTSNS..NE**F**NPARIT

95   143 147   250   254

C

P252 S147 Y145 Y250

D

○ 1
○ 2-10
○ >10

● non-AHC strains (1951-now)
● 1st variant isolate
● 1st pandemic or later (1985-2001)
● 2nd pandemic or later (2002-now)

Finland, Russia, Spain, China, Japan, South Korea, USA, Puerto Rico, Jamaica, India, Thailand, Taiwan, Malaysia, Cambodia, Cameroon, Gabon, Brazil, Réunion, South Africa, Australia

1st AHC outbreak Singapore 1970

E

	number of strains	Sia-binding site		

CV-A24 no AHC: 37 — F RYYTS FNPAR
1st variant isolate (EH24/70) AHC: 1 — F RYYTS FNPAR
variant AHC: 188 — F RYYAS YNPAR

95 143 144 145 146 147 250 251 252 253 254

F

HeLa / HCE / HCE + NA
WT
Y250F

HeLa: WT 8±, Y250F 8± ns
HCE: WT ~24, Y250F ~11 ****
HCE + NA: ns

G

Binding to HCE cells

Relative RNA level

WT / Y250F (mock) ** ; WT / Y250F (NA) ns